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(54) Title: PEPTIDE INHIBITORS OF SELECTIN BINDING (57) Abstract The present invention provides peptides comprising portions of the amino acid sequence at positions 58-61 of P-selectin. The invention also provides pharmaceutical compositions comprising the peptides of the invention, diagnostic and therapeutic methods utilizing the peptides and pharmaceutical compositions of the invention, and method of preparing the peptides and pharmaceutical compositions.		

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PEPTIDE INHIBITORS OF SELECTIN BINDING

Background of the Invention

This invention relates to peptides which inhibit binding of selectins such as P-selectin, E-selectin and L-selectin.

The adherence of platelets and leukocytes to vascular surfaces is a critical component of the inflammatory response and is part of a complex series of reactions involving the simultaneous and interrelated activation of the complement, coagulation, and immune systems.

The complement proteins collectively play a leading role in the immune system, both in the identification and in the removal of foreign substances and immune complexes, as reviewed by Muller-Eberhard, H.J., Ann. Rev. Biochem. 57: 321-347 (1988). Central to the complement system are the C3 and C4 proteins, which when activated covalently attach to nearby targets, marking them for clearance. In order to help control this process, a remarkable family of soluble and membrane-bound regulatory proteins has evolved, each of which interacts with activated C3 and/or C4 derivatives. The coagulation and inflammatory pathways are regulated in a coordinate fashion in response to tissue damage. For example, in addition to becoming adhesive for leukocytes, activated endothelial cells express tissue factor on the cell surface and decrease their surface expression of thrombomodulin, leading to a net facilitation of coagulation reactions on the cell surface. In some cases, a single

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recept r can be involved in both inflammatory and coagulation processes.

Leukocyte adherence to vascular endothelium is a key initial step in migration of leukocytes to tissues in response to microbial invasion. Although a class of inducible leukocyte receptors, the CD11-CD18 molecules, are thought to have some role in adherence to endothelium, mechanisms of equal or even greater importance for leukocyte adherence appear to be due to inducible changes in the endothelium itself.

Activated platelets have also been shown to interact with both neutrophils and monocytes *in vitro*. The interaction of platelets with monocytes may be mediated in part by the binding of thrombospondin to platelets and monocytes, although other mechanisms have not been excluded. The mechanisms for the binding of neutrophils to activated platelets are not well understood, except that it is known that divalent cations are required. In response to vascular injury, platelets are known to adhere to subendothelial surfaces, become activated, and support coagulation. Platelets and other cells may also play an important role in the recruitment of leukocytes into the wound in order to contain microbial invasion.

Endothelium exposed to "rapid" activators such as thrombin and histamine becomes adhesive for neutrophils within two to ten minutes, while endothelium exposed to cytokines such as tumor necrosis factor and interleukin-1 becomes adhesive after one to six hours. The rapid endothelial-dependent leukocyte adhesion has been associated with expression of the lipid mediator platelet activating factor (PAF) on the cell surface, and presumably, the appearance of other endothelial surface molecules. The slower cytokine-inducible endothelial adhesion for leukocytes is mediated, at least in part by E-selectin that is synthesized by endothelial cells after exposure to cytokines and then transported to the cell surface, where it binds neutrophils. The isolation, characterization and cloning of

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E-selectin, also known as ELAM-1, is reviewed by Bvilacqua, et al., in Science 243, 1160-1165 (1989). L-selectin, also known as peripheral lymph node homing receptor, "the murine Mel 14 antigen", "Leu 8", the "Leu 8 antigen" and "LAM-1", is another structure on neutrophils, monocytes, and lymphocytes that binds lymphocytes to high endothelial venules in peripheral lymph nodes. The characterization and cloning of the protein is reviewed by Lasky, et al., Cell 56, 1045-1055 (1989) (mouse) and Tedder, et al., J. Exp. Med. 170, 123-133 (1989).

P-selectin, also known as GMP-140 (granule membrane protein 140) or PADGEM, is a cysteine-rich and heavily glycosylated integral membrane glycoprotein with an apparent molecular weight of 140,000 as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). P-selectin was first purified from human platelets by McEver and Martin, J. Biol. Chem. 259: 9799-9804 (1984). The protein is present in alpha granules of resting platelets but is rapidly redistributed to the plasma membrane following platelet activation, as reported by Stenberg, et al., (1985). The presence of P-selectin in endothelial cells and its biosynthesis by these cells was reported by McEver, et al., Blood 70(5) Suppl. 1:355a, Abstract No. 1274 (1987). In endothelial cells, P-selectin is found in storage granules known as the Weibel-Palade bodies. (McEver, et al. J. Clin. Invest. 84: 92-99 (1989) and Hattori, et al., J. Biol. Chem. 264: 7768-7771 (1989)). P-selectin (also called GMP-140 or PADGEM) has also been reported to mediate the interaction of activated platelets with neutrophils and monocytes by Larsen, et al., in Cell 59, 305-312 (October 1989) and Hamburger and McEver, Blood 75: 550-554 (1990).

The cDNA-derived amino acid sequence, reported by Johnston, et al., in Cell 56, 1033-1044 (March 24 1989), and in U.S. Serial No. 07/320,408 filed March 8, 1989, indicates that it contains a number of modular domains that are likely to fold independently. Beginning at the N-terminus, these include a "lectin" domain, an "EGF" domain, nine tandem

consensus repeats similar to those in complement binding proteins, a transmembrane domain (except in a soluble form that appears to result from differential splicing), and a cytoplasmic tail.

5 When platelets or endothelial cells are activated by mediators such as thrombin, the membranes of the storage granules fuse with the plasma membrane, the soluble contents of the granules are released to the external environment, and membrane bound P-selectin is presented within seconds on the
10 cell surface. The rapid redistribution of P-selectin to the surface of platelets and endothelial cells as a result of activation suggested that this glycoprotein could play an important role at sites of inflammation or vascular disruption.

15 This important role has been confirmed by the observation that P-selectin is a receptor for neutrophils (Geng et al., Nature 343:757-760 (1990); Hamburger and McEver, Blood 75:550-554 (1990)), monocytes (Larsen, et al. Cell 59:305-312 (1989)); Moore, et al., J. Cell Biol.
20 112:491-499 (1991)), and perhaps a subset of lymphocytes (Moore, et al. J. Cell Biol. 112:491-499 (1991)). Thus, P-selectin can serve as a receptor for leukocytes following its rapid mobilization to the surfaces of platelets and endothelial cells stimulated with agonists such as thrombin.
25 This role in leukocyte recruitment may be important in hemostatic and inflammatory processes in both physiologic and pathologic states.

 Peptides derived from P-selectin are described in U.S. Serial No. 07/554,199 entitled "Functionally Active
30 Selectin-Derived Peptides" filed July 17, 1990 by Rodger P. McEver that are useful in diagnostics and in modulating the hemostatic and inflammatory responses in a patient wherein a therapeutically effective amount of a peptide capable of blocking leukocyte recognition of P-selectin is administered
35 to the patient. U.S. Serial No. 07/554,199 filed July 17, 1990 also discloses that peptide sequences within the lectin domain of P-selectin, having homology with the lectin domains

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of other proteins, especially E-selectin and L-selectin, selectively inhibit neutrophil adhesion to purified P-selectin, and can therefore be used in diagnostic assays of patients and diseases characterized by altered binding by these molecules, in screening assays for compounds altering this binding, and in clinical applications to inhibit or modulate interactions of leukocytes with platelets or endothelial cells involving coagulation and/or inflammatory processes.

10 P-selectin, E-selectin, and L-selectin constitute the selectin family, based on their related structure and function. E-selectin is not present in unstimulated endothelium. However, when endothelium is exposed to cytokines such as tumor necrosis factor or interleukin-1, the
15 gene for E-selectin is transcribed, producing RNA which in turn is translated into protein. The result is that E-selectin is expressed on the surface of endothelial cells one to four hours after exposure to cytokines, as reported by Bevilacqua et al., Proc.Natl.Acad.Sci.USA 84: 9238-9242
20 (1987) (in contrast to P-selectin, which is stored in granules and presented on the cell surface within seconds after activation). E-selectin has been shown to mediate the adherence of neutrophils to cytokine-treated endothelium and thus appears to be important in allowing leukocytes to
25 migrate across cytokine-stimulated endothelium into tissues. The cDNA-derived primary structure of E-selectin indicates that it contains a "lectin" domain, an EGF domain, and six (instead of the nine in GMP-140) repeats similar to those of complement-regulatory proteins, a transmembrane domain, and a
30 short cytoplasmic tail. There is extensive sequence homology between P-selectin and E-selectin throughout both proteins, but the similarity is particularly striking in the lectin and EGF domains.

Homing receptors are lymphocyte surface structures
35 that allow lymphocytes to bind to specialized endothelial cells in lymphatic tissues, termed high endothelial cells or high endothelial venules (reviewed by Yednock and Rose,

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Advances in Immunology, vol. 44, F.I. Dixon, ed., 313-378 (Academic Press, New York 1989). This binding allows lymphocytes to migrate across the endothelium into the lymphatic tissues where they are exposed to processed antigens. The lymphocytes then re-enter the blood through the lymphatic system. L-selectin contains a lectin domain, an EGF domain, two complement-binding repeats, a transmembrane domain, and a short cytoplasmic tail. L-selectin also shares extensive sequence homology with P-selectin, particularly in the lectin and EGF domains.

Based on a comparison of the lectin domains between P-, E- and L-selectin, it may be possible to select those peptides inhibiting binding of neutrophils to P-selectin which will inhibit binding of E-selectin, L-selectin and other homologous molecules, to components of the inflammatory process, or, conversely, which will inhibit only one selectin-mediated binding.

The *in vivo* significance of platelet-leukocyte interactions has not been studied carefully. However, in response to vascular injury, platelets are known to adhere to subendothelial surfaces, become activated, and support coagulation. Platelets and other cells may also play an important role in the recruitment of leukocytes into the wound in order to contain microbial invasion. Conversely, leukocytes may recruit platelets into tissues at sites of inflammation, as reported by Issekutz, et al., Lab. Invest. 49:716 (1983).

The coagulation and inflammatory pathways are regulated in a coordinate fashion in response to tissue damage. For example, in addition to becoming adhesive for leukocytes, activated endothelial cells express tissue factor on the cell surface and decrease their surface expression of thrombomodulin, leading to a net facilitation of coagulation reactions on the cell surface. In some cases, a single receptor can be involved in both inflammatory and coagulation processes.

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Proteins involved in the hemostatic and inflammatory pathways are of interest for diagnostic purposes and treatment of human disorders. However, there are many problems using proteins therapeutically. Proteins are usually expensive to produce in quantities sufficient for administration to a patient. Moreover, there can be a reaction against the protein after it has been administered more than once to the patient. It is therefore desirable to develop peptides having the same, or better, activity as the protein, which can be synthesized in quantity, and are not immunogenic.

It is preferable to develop peptides which can be prepared synthetically, having activity at least equal to, or greater than, peptide sequences contained within the proteins themselves.

It is therefore an object of the present invention to provide peptides interacting with cells recognized by selectins, including P-selectin, E-selectin, and L-selectin.

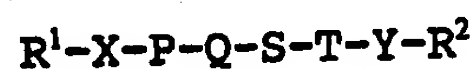
It is another object of the present invention to provide methods for using these peptides to inhibit leukocyte adhesion to endothelium or to platelets.

It is a further object of the present invention to provide methods for using these peptides to modulate the immune response and the hemostatic pathway.

It is yet another object of the present invention to provide peptides for use in diagnostic assays relating to GMP-140, P-, E- or L-selectin.

Summary of the Invention

This invention relates to novel peptides of the formula:



(I)

or pharmaceutically acceptable salts thereof, where:

X is an N-terminus amino acid linear chain of from 0 to 10 amino acids, and R^1 is a moiety attached to the

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terminal α -amino group of X or the α -amino group of P if X is zero, or the α -amino group of Q if X is zero and P is desamino acid;

Y is a C-terminal amino acid linear chain of from 0 to 10 amino acids, and R^2 is a moiety attached to the carbon of the carboxyl function of Y ($C(O)R^2$) or the carbon of the carboxyl function of T if Y is zero;

P is D- or L-lysine, D- or L- ϵ -acetyl-lysine, D- or L-asparagine, glycine, D- or L-valine, D- or L-glutamine, D- or L-glutamic acid, D- or L-alanine, or a desamino acid, where desamino acid signifies no amino acid is present at this point in the formula;

Q is an amino acid selected from the group consisting of D- or L-threonine, D- or L-isoleucine, D- or L-valine, D- or L-alanine, D- or L-glutamine;

S is an amino acid selected from the group consisting of D- or L-tryptophan, D- or L-glutamine, D- or L-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, D- or L- ϵ -acetyl-lysine;

T is an amino acid selected from the group consisting of D- or L-threonine, D- or L-valine, D- or L-alanine, D- or L-glutamine, D- or L- ϵ -acetyl-lysine;

R^1 is hydrogen (signifying a free N-terminal group), lower alkyl, aryl, formyl, alkanoyl, aroyl, alkyloxycarbonyl or aroyloxycarbonyl;

R^2 is OH (signifying a free C-terminal carboxylic acid), OR^3 , signifying esters, where R^3 is lower alkyl or aryl, or R^2 is NR^4R^5 , where R^4 and R^5 are each selected independently from hydrogen, lower alkyl, aryl or cyclic alkyl;

provided that X is not equal to Z-A-B-, where Z is a sequence of 0-8 amino acids; A is an amino acid selected from the group consisting of D- or L-asparagine, D- or L-isoleucine, and D- or L-valine; and B is an amino acid selected from the group consisting of D- or L-asparagine and glycine.

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Peptides of Formula I have as their core region portions of the 58-61 amino acid sequence of P-selectin, with residue 1 defined as the N-terminus of the mature protein after the cleavage of the signal peptide.

5 Tests indicate that the peptides of Formula I inhibit the binding of neutrophils to P-selectin in concentrations of peptide ranging from about 50 to about 1500 μ M. Tests also indicate that alterations within the core sequence, as well as N-terminal and C-terminal flanking
10 regions, do not result in loss of biological activity.

This invention relates not only to the novel peptides of Formula I, but also to pharmaceutical compositions comprising them, to diagnostic and therapeutic methods utilizing them, and to methods of preparing them.

15 Brief Description of the Drawings

Figure 1 shows the peptides of Formula I to inhibit the adhesion of human neutrophils to purified human P-selectins.

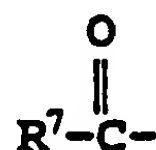
Detailed Description of the Invention

20 Preferred peptides of this invention are those of Formula I wherein, together or independently: R¹ is hydrogen; R² is NH₂; S is D- or L-tryptophan; and T is D- or L-threonine. Other preferred peptides of this invention are those of Formula I wherein, together or independently: X is
25 selected from the group consisting of Asn, D-Asn, Gly-Gln, D-Gln, ϵ -acetyl-lysine and Y is selected from the group consisting of Gln-Val, Gln-D-Val, D-Gln-Val, D-Gln-D-Val, Trp-Gln, D-Trp-Gln, Trp-D-Gln, D-Trp-D-Gln, 7-hydroxy-1,2,3,4-tetrahydroisoquiniline-3-carboxylic acid-Gln, D-7-
30 hydroxy-1,2,3,4-tetrahydroisoquiniline-3-carboxylic acid-Gln, D-7-hydroxy-1,2,3,4-tetrahydroisoquiniline-3-carboxylic acid-Gln, D-7-hydroxy-1,2,3,4-tetrahydroisoquiniline-3-carboxylic acid-D-Gln, ϵ -acetyl-lysine-Gln, D- ϵ -acetyl-lysine-Gln, ϵ -acetyl-lysine-D-Gln, D- ϵ -acetyl-lysine-D-Gln.

Specifically preferred peptides include the following:

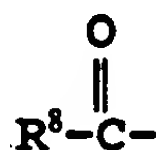
- Gly-Ile-Trp-Thr-Trp-Val (SEQ ID. NO: 1)
 Gly-Gly-Ile-Trp-Thr-Trp-Val (SEQ ID. NO: 2)
 5 Gly-Gly-D-Ile-D-Trp-D-Thr-D-Trp-D-Val (SEQ ID. NO: 3)
 Gly-D-Ile-D-Trp-D-Thr-D-Trp-D-Val (SEQ ID. NO: 4)
 Asn-Lys-Thr-Trp-Thr-Trp-Val-NH₂ (SEQ ID. NO: 5)
 Lys-Thr-Trp-Thr-Trp-Val-NH₂ (SEQ ID. NO: 6)
 10 Thr-Trp-Thr-Trp-Val-NH₂ (SEQ ID. NO: 7)

As used herein, the term "alkyl" includes branched, straight-chain, and cyclic saturated hydrocarbons. The term "lower alkyl" means an alkyl having from one to six carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl,
 15 isobutyl, t-butyl, pentyl, isopentyl, neopentyl, cyclopentylmethyl and hexyl. The term "alkanoyl" means



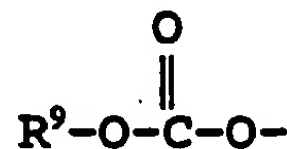
20 wherein R⁷ is a alkyl group.

The term "aroyl" means



25 wherein R⁸ is an aryl group. The term "aryl" means an aromatic or heteroaromatic structure having between one and three rings, which may or may not be ring fused structures, and are optimally substituted with halogens, carbons, or other heteroatoms such as nitrogen (N), sulfur
 30 (S), phosphorus (P), and boron (B).

The term alkoxycarbonyl means



35 wherein R⁹ is an aryl and arylmethyl group.

The term "terminal α-amino group of X" refers to the α-amin group of the N-terminal amino acid of X.

The peptides of formula I can be used in the form of the free peptide or a pharmaceutically acceptable salt.

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Amine salts can be prepared by treating the peptide with an acid according to known methods. Suitable acids include inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalenesulfonic acid, and sulfanilic acid.

10 Carboxylic acid groups in the peptide can be converted to a salt by treating the peptide with a base according to known methods. Suitable bases include inorganic bases such as sodium hydroxide, ammonium hydroxide, and potassium hydroxide, and organic bases such as mono-, di-,
15 and tri-alkyl and aryl amines (e.g., triethylamine, diisopropylamine, methylamine, and dimethylamine and optionally substituted mono-, di, and tri-ethanolamines.

As referred to herein, the amino acid components of the peptides and certain materials used in their preparation
20 are identified by abbreviations for

convenience. These abbreviations are as follows:

Amin Acid	Abbr viati ns
L-alanine	Ala A
D-alanine	D-Ala a
5 L-arginine	Arg R
D-arginine	D-Arg r
D-asparagine	D-Asn N
L-asparagine	L-Asn n
L-aspartic acid	Asp D
10 D-aspartic acid	D-Asp d
L-cysteine	Cys C
D-cysteine	D-Cys c
L-glutamic acid	Glu E
D-glutamic acid	D-Glu e
15 L-glutamine	Gln Q
D-glutamine	D-Gln q
glycine	Gly G
L-histidine	His H
D-histidine	D-His h
20 L-isoleucine	Ile I
D-isoleucine	D-Ile i
L-leucine	Leu L
D-leucine	D-Leu l
L-lysine	Lys K
25 D-lysine	D-Lys k
L-phenylalanine	Phe F
D-phenylalanine	D-Phe f
L-proline	Pro P
D-proline	D-Pro p
30 L-pyroglutamic acid	pGlu
D-pyroglutamic acid	DpGlu
L-serine	L-Ser S
D-serine	D-Ser s
L-threonine	L-Thr T
35 D-threonine	D-Thr t
L-tyrosine	L-Tyr Y
D-tyrosine	D-Tyr y
L-tryptophan	Trp W
D-tryptophan	D-Trp w
40 L-valine	Val V
D-valine	D-Val v
ϵ -acetyl-lysine	Lys(Ac)
7-hydroxy-1,	Tic(OH)
2,3,4-tetrahydro-	
45 isoquiniline-3-	
carboxylic acid	
D-7-hydroxy-1,	D-Tic(OH)
2,3,4-tetrahydro-	
50 isoquiniline-3-	
carboxylic acid	

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ReagentsAbbreviations

	Trifluoroacetic acid	TFA
	Methyl ne chloride	CH ₂ Cl ₂
	N,N-Diisopropylethylamine	DIEA
5	N-Methylpyrrolidone	NMP
	1-Hydroxybenzotriazole	HOBT
	Dimethylsulfoxide	DMSO
	Acetic anhydride	Ac ₂ O
	Diisopropylcarbodiimide	Dic

10 Amino acids preceded by L- or D- refer, respectively, to the L- or D- enantiomer of the amino acid, whereas amino acids not preceded by L- or D- refer to the L-enantiomer.

Methods of Preparation of Peptides

15 The peptides can generally be prepared following known techniques, as described, for example, in the cited publications, the teachings of which are specifically incorporated herein. In a preferred method, the peptides are prepared following the solid-phase synthetic technique
 20 initially described by Merrifield in J.Amer.Chem.Soc., 85, 2149-2154 (1963). Other techniques may be found, for example, in M. Bodanszky, et al, Peptide Synthesis, second edition, (John Wiley & Sons, 1976), as well as in other reference works known to those skilled in the art.

25 Appropriate protective groups usable in such syntheses and their abbreviations will be found in the above text, as well as in J.F.W. McOmie, Protective Groups in Organic Chemistry, (Plenum Press, New York, 1973). The common protective groups used herein are t-butyloxycarbonyl
 30 (Boc), fluorenylmethoxycarbonyl (FMOC), benzyl (Bzl), tosyl (Tos), o-bromo-phenylmethoxycarbonyl (BrCBZ), phenylmethoxycarbonyl (CBZ), 2-chloro-phenylmethoxycarbonyl (2-Cl-CBZ), 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr), trityl (Trt), formyl (CHO), and tertiary butyl (t-Bu).

35 General synthetic procedures for the synthesis of peptides of Formula I by solid phase methodology are as follows:

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A. General Synthetic Procedures For Solid Phase Peptide Synthesis Using N^α-Boc Protection

			<u>REPETITIONS</u>	<u>TIME</u>
5	1.	25% TFA in CH ₂ Cl ₂	1	3 min.
	2.	50% TFA in CH ₂ Cl ₂	1	16 min.
	3.	CH ₂ Cl ₂	5	3 min.
	4.	5% DIEA in NMP	2	4 min.
	5.	NMP	6	5 min.
10	6.	Coupling step	1	57 min.
	a.	Preformed BOC-Amino Acid-HOBT active ester in NMP		37 min.
	b.	DMSO		16 min.
	c.	DIEA		5 min.
15	7.	10% Ac ₂ O, 5% DIEA in NMP	1	9 min.
	8.	CH ₂ Cl ₂	5	3 min.

B. General Synthetic Procedure For Solid Phase Peptide Synthesis Using N^α-Fmoc Protection

			<u>REPETITIONS</u>	<u>TIME</u>
20	1.	20% piperidine in NMP	1	3 min.
	2.	20% piperidine in NMP	1	15 min.
	3.	NMP	6	9 min.
	4.	Coupling	1	71 min.
		Preformed Fmoc-Amino Acid-HOBT active ester in NMP or Fmoc-amino acid and HOBT in NMP followed by the addition of DIC in NMP		
25	5.	NMP	6	7 min.

30 The peptides can also be prepared using standard genetic engineering techniques known to those skilled in the art. For example, the peptide can be produced enzymatically by inserting nucleic acid encoding the peptide into an expression vector, expressing the DNA, and translating the

35 DNA into the peptide in the presence of the required amino

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acids. The peptide is then purified using chromatographic or electrophoretic techniques, or by means of a carrier protein which can be fused to, and subsequently cleaved from, the peptide by inserting into the expression vector in phase with the peptide encoding sequence a nucleic acid sequence encoding the carrier protein. The fusion protein-peptide may be isolated using chromatographic, electrophoretic or immunological techniques (such as binding to a resin via an antibody to the carrier protein). The peptide can be cleaved using chemical methodology or enzymatically, as by, for example, hydrolases.

Peptides of Formula I can also be prepared using solution methods, by either stepwise or fragment condensations. An appropriately amino-terminal-protected amino acid is coupled to an appropriately carboxyl-terminal protected amino acid (such protection may not be required depending on the coupling method chosen) using diimides, symmetrical or unsymmetrical anhydrides, BOP, or other coupling reagents or techniques known to those skilled in the art. These techniques may be either chemical or enzymatic. The amino and/or carboxyl protecting groups are removed and the next suitably protected amino acid or block of amino acids is coupled to extend the growing peptide. Various combinations of protecting groups and of chemical and/or enzymatic techniques and assembly strategies can be used in each synthesis.

Methods of Preparation of Pharmaceutical Compositions

Pharmaceutical compositions of this invention comprise a pharmaceutically acceptable carrier or diluent and an effective quantity of one or more of the peptides of Formula I, or an acid or base salt thereof. The carrier or diluent may take a wide variety of forms depending on the form of preparation desired for administration, e.g., sublingual, rectal, nasal, oral, or parenteral.

In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, for

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exampl , waters, oils, alcohols, flavoring ag nts,
preservatives, and coloring agents, to make an oral liquid
preparation (.g., suspension, elixir, or solution) or with
carriers such as starches, sugars, diluents, granulating
5 agents, lubricants, binders, and disintegrating agents, to
make an oral solid preparation (e.g., powder, capsule, or
tablet).

Controlled release forms or enhancers to increase
bioavailability may also be used. Because of their ease in
10 administration, tablets and capsules represent the most
advantageous oral dosage unit form, in which case solid
pharmaceutical carriers are employed. If desired, tablets
may be sugar coated or enteric coated by standard techniques.

For parenteral products, the carrier will usually
15 be sterile water, although other ingredients to aid
solubility or as preservatives may be included. Injectable
suspensions may also be prepared, in which case appropriate
liquid carriers and suspending agents can be employed.

The peptides can also be administered locally at a
20 wound or inflammatory site by topical application of a
solution or cream.

Alternatively, the peptide may be administered in
liposomes or microspheres (or microparticles). Methods for
preparing liposomes and microspheres for administration to a
25 patient are known to those skilled in the art. U.S. Patent
No. 4,789,734 describes methods for encapsulating biological
materials in liposomes. Essentially, the material is
dissolved in an aqueous solution, the appropriate
phospholipids and lipids added, along with surfactants if
30 required, and the material dialyzed or sonicated, as
necessary. A review of known methods is by G. Gregoriadis,
Chapter 14, "Liposomes", Drug Carriers in Biology and
Medicine, pp. 287-341 (Academic Press, 1979). Microspheres
formed of polymers or proteins are well known to those
35 skilled in the art, and can be tailored for passage through
th gastrointestinal tract directly into the bloodstream.
Alternatively, the peptide can be incorporated and the

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microspher s, or composite of microspher s, implanted for slow r lease over a period of time, ranging from days to months. S , for example, U.S. Patents Nos. 4,906,474, 4,925,673 and 3,625,214.

5 The peptides are generally active when administered parenterally in amounts above about 1 μ g/kg body weight. Effective doses by other routes of administration are generally those which result in similar blood level to i.v. doses above about 1 μ g/kg. For treatment to prevent organ
10 injury in cases involving reperfusion, the peptides may be administered parenterally in amounts from about 0.01 to about 10 mg/kg body weight. Generally, the same range of dosage amounts may be used in treatment of other diseases or of conditions where inflammation is to be reduced. This dosage
15 will be dependent, in part, on whether one or more peptides are administered. A synergistic effect may be seen with combinations of peptides from different, or overlapping, regions of the lectin domain, or in combination with peptides derived form the EGF domain of P-selectin.

20 **Methods for Demonstrating Binding**

 Peptides that are biologically active are those which inhibit binding of neutrophils, monocytes, subsets of lymphocytes or other cells to P-selectin, or which inhibit leukocyte adhesion to endothelium that is mediated by E-
25 selectin and/or L-selectins.

 Peptides can be screened for their ability to inhibit adhesion to cells, for example, neutrophil adhesion to purified P-selectin immobilized on plastic wells, using the assay described by Geng, et al., Nature 343, 757-760
30 (1990).

 Human neutrophils are isolated from heparinized whole blood by density gradient centrifugation on Mono-Poly resolving media, Flow Laboratories. Neutrophil suspensions are greater than 98% pure and greater than 95% viable by
35 trypan blue exclusion. For adhesion assays, neutrophils are suspended at a conc ntration of 2×10^6 cells/mL in Hanks' balanced salt solution containing 1.26 mM Ca^{2+} and 0.81 mM

Mg²⁺ (HBSS, Gibco) with g mg/mL human serum albumin (HBSS/HSA). Adhesion assays are conducted in triplicate in 96-well microtiter plates, Corning, incubated at 4°C overnight with 50 microliters of various protein solutions.

5 P-selectin is isolated from human platelet lysates by immunoaffinity chromatography on antibody S12-Sepharose™ and ion-exchange chromatography on a Mono-Q™ column (FLPC, Pharmacia Fine Chemicals), as follows.

Outdated human platelet packs (100 units) obtained
10 from a blood bank and stored at 4°C are pooled, adjusted to 5mM EDTA at pH 7.5, centrifuged at 4,000 rpm for 30 minutes in 1 liter bottles, then washed three times with 1 liter of 0.1 M NaCl, 20 mM Tris pH 7.5 (TBS), 5 mM EDTA, 5 mM benzamidine.

15 The pellets are then resuspended in a minimum amount of wash buffer and made 1mM in DIFP, then frozen in 50 mL screwtop tubes at -80°C. The frozen platelets are thawed and resuspended in 50 mL TBS, 5 mM benzamidine, 5 mM EDTA pH 7.5, 100 M leupeptin. The suspension is frozen and thawed
20 two times in a dry ice-acetone bath using a 600 mL lyophilizing flask, then homogenized in a glass/teflon mortar and pestle and made 1 mM in DIFP. The NaCl concentration is adjusted to 0.5 M with a stock solution of 4 M NaCl. After stirring the suspension at 4°C, it is centrifuged in
25 polycarbonate tubes at 33,000 rpm for 60 minutes at 4°C. The supernatant (0.5 M NaCl wash) is removed and saved; this supernatant contains the soluble form of P-selectin. Care is taken not to remove the top part of the pellet with the supernatant. The pellets are then homogenized in extraction
30 buffer (TBS, 5 mM benzamidine, 5 mM EDTA, pH 7.5, 100 μM leupeptin, 2% Triton X-100). After centrifugation at 19,500 rpm for 25 minutes at 4°C, the supernatant is removed. The extraction procedure is repeated with the pellet and the supernatant is combined with the first supernatant. The
35 combined extracts, which contain the membrane form of P-selectin, are adjusted to 0.5 M NaCl.

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The soluble fraction (0.5 M NaCl wash) and the membrane extract (also adjusted to 0.5 M NaCl) are absorbed with separate pools of the monoclonal antibody S12 (directed to P-selectin) previously coupled to Affigel (Biorad) at 5 mg/mL for 2 hours at 4°C. After letting the resins settle, the supernatants are removed. The S12 Affigel containing bound GMP-140 is then loaded into a column and washed overnight at 4°C with 400 mL of 0.5 M NaCl, 20 mM Tris pH 7.5, 0.01% Lubrol PX.

10 Bound P-selectin is eluted from the S12 Affigel with 100 mL of 80% ethylene glycol, 1 mM MES pH 6.0, 0.01% Lubrol PX. Peak fractions with absorbance at 280 nm are pooled. Eluates are dialyzed against TBS with 0.05% Lubrol, then applied to a Mono Q column (FPLC from Pharmacia). The
15 concentrated protein is step eluted with 2 M NaCl, 20 mM Tris pH 7.5 (plus 0.05% Lubrol PX for the membrane fraction). Peak fractions are dialyzed into TBS pH 7.5 (plus 0.05% Lubrol PX for the membrane fraction).

P-selectin is plated at 5 micrograms/mL and the
20 control proteins: human serum albumin (Alb), platelet glycoprotein IIb/IIIa (IIb), von Willebrand factor (vWF), fibrinogen (FIB), thrombomodulin (TM), gelatin (GEL) or human serum (HS), are added at 50 micrograms/mL. All wells are blocked for 2 hours at 22°C with 300 microliters HBSS
25 containing 10 mg/mL HSA, then washed three times with HBSS containing 0.1% Tween-20 and once with HBSS. Cells (2×10^5 per well) are added to the wells and incubated at 22°C for 20 minutes. The wells are then filled with HBSS/HSA, sealed with acetate tape (Dynatech), and centrifuged inverted at 150
30 g for 5 minutes. After discarding nonadherent cells and supernates, the contents of each well are solubilized with 200 microliters 0.5% hexadecyltrimethylammonium bromide, Sigma, in 50 mM potassium phosphate, pH. 6.0, and assayed for myeloperoxidase activity, Ley, et al., Blood 73, 1324-1330
35 (1989). The number of cells bound is derived from a standard curve of myeloperoxidase activity versus numbers of cells. Under all assay conditions, the cells release less than 5% of

total myeloperoxidase and lactate dehydrogenase. Inhibition is read as a lower percent adhesion, so that a value of 5% means that 95% of the specific adhesion was inhibited.

Clinical Applications

5 Since the selectins have several functions related to leukocyte adherence, inflammation, and coagulation, compounds which interfere with binding of P-selectin, E-selectin or L-selectin can be used to modulate these responses.

10 For example, the peptides can be used to competitively inhibit leukocyte adherence by competitively binding to P-selectin receptors on the surface of leukocytes. This kind of therapy would be particularly useful in acute situations where effective, but transient, inhibition of
15 leukocyte-mediated inflammation is desirable. Chronic therapy by infusion of the peptides may also be feasible in some circumstances.

 An inflammatory response may cause damage to the host if unchecked, because leukocytes release many toxic
20 molecules that can damage normal tissues. These molecules include proteolytic enzymes and free radicals. Examples of pathological situations in which leukocytes can cause tissue damage include injury from ischemia and reperfusion, bacterial sepsis and disseminated intravascular coagulation,
25 adult respiratory distress syndrome, tumor metastasis, rheumatoid arthritis and atherosclerosis.

 Reperfusion injury is a major problem in clinical cardiology. Therapeutic agents that reduce leukocyte adherence in ischemic myocardium can significantly enhance
30 the therapeutic efficacy of thrombolytic agents. Thrombolytic therapy with agents such as tissue plasminogen activator or streptokinase can relieve coronary artery obstruction in many patients with severe myocardial ischemia prior to irreversible myocardial cell death. However, many
35 such patients still suffer myocardial necrosis despite restoration of blood flow. This "reperfusion injury" is known to be associated with adherence of leukocytes to

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vascular endothelium in the ischemic zone, presumably in part because of activation of platelets and endothelium by thrombin and cytokines that makes them adhesive for leukocytes (Romson et al., Circulation 67: 1016-1023 (1983)).

- 5 These adherent leukocytes can migrate through the endothelium and destroy ischemic myocardium just as it is being rescued by restoration of blood flow.

There are a number of other common clinical disorders in which ischemia and reperfusion results in organ injury mediated by adherence of leukocytes to vascular surfaces, including strokes; mesenteric and peripheral vascular disease; organ transplantation; and circulatory shock (in this case many organs might be damaged following restoration of blood flow).

- 15 Bacterial sepsis and disseminated intravascular coagulation often exist concurrently in critically ill patients. They are associated with generation of thrombin, cytokines, and other inflammatory mediators, activation of platelets and endothelium, and adherence of leukocytes and aggregation of platelets throughout the vascular system. 20 Leukocyte-dependent organ damage is an important feature of these conditions.

Adult respiratory distress syndrome is a devastating pulmonary disorder occurring in patients with sepsis or following trauma, which is associated with widespread adherence and aggregation of leukocytes in the pulmonary circulation. This leads to extravasation of large amounts of plasma into the lungs and destruction of lung tissue, both mediated in large part by leukocyte products.

- 30 Two related pulmonary disorders that are often fatal are in immunosuppressed patients undergoing allogeneic bone marrow transplantation and in cancer patients suffering from complications that arise from generalized vascular leakage resulting from treatment with interleukin-2 treated LAK cells (lymphokine-activated lymphocytes). LAK cells are 35 known to adhere to vascular walls and release products that are presumably toxic to endothelium. Although the mechanism

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by which LAK cells adhere to endothelium is now known, such cells could potentially release molecules that activate endothelium and then bind to endothelium by mechanisms similar to those operative in neutrophils.

5 Tumor cells from many malignancies (including carcinomas, lymphomas, and sarcomas) can metastasize to distant sites through the vasculature. The mechanisms for adhesion of tumor cells to endothelium and their subsequent migration are not well understood, but may be similar to
10 those of leukocytes in at least some cases. The association of platelets with metastasizing tumor cells has been well described, suggesting a role for platelets in the spread of some cancers. Recently, it was reported that P-selectin binds to tumor cells in a variety of human carcinoma tissue
15 sections (colon, lung, and breast), and that P-selectin binds to the cell surface of a number of cell lines derived from various carcinomas, but not from melanomas. Aruggo, A., et al., Proc. Natl. Acad. Sci. USA, 89, 2292-2296 (1992). Aruggo et al. also reference earlier work suggesting that E-
20 selectin might be involved in tumor metastasis by mediating the adhesion of a colon carcinoma cell line (HT-20) to activated endothelial cells in vitro. Platelet-leukocyte interactions are believed to be important in atherosclerosis. Platelets might have a role in recruitment of monocytes into
25 atherosclerotic plaques; the accumulation of monocytes is known to be one of the earliest detectable events during atherogenesis. Rupture of a fully developed plaque may not only lead to platelet deposition and activation and the promotion of thrombus formation, but also the early
30 recruitment of neutrophils to an area of ischemia.

Another area of potential application is in the treatment of rheumatoid arthritis.

The criteria for assessing response to therapeutic modalities employing these peptides, and, hence, effective
35 dosages of the peptides of this invention for treatment, are dictated by the specific condition and will generally follow standard medical practices. For example, the criteria for

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th effective dosag to prevent extension of myocardial infarction would b d terminated by one skilled in th art by looking at mark r enzymes of myocardial n crosis in th plasma, by monitoring the electrocardiogram, vital signs, and clinical response. For treatment of acute respiratory distress syndrome, one would examine improvements in arterial oxygen, resolution of pulmonary infiltrates, and clinical improvement as measured by lessened dyspnea and tachypnea. For treatment of patients in shock (low blood pressure), the effective dosage would be based on the clinical response and specific measurements of function of vital organs such as the liver and kidney following restoration of blood pressure. Neurologic function would be monitored in patients with stroke. Specific tests are used to monitor the functioning of transplanted organs; for example, serum creatinine, urine flow, and serum electrolytes in patients undergoing kidney transplantation.

Diagnostic Reagents

The peptides can also be used for the detection of human disorders in which the ligands for the selectins might be defective. Such disorders would most likely be seen in patients with increased susceptibility to infections in which leukocytes might not be able to bind to activated platelets or endothelium. Cells to be tested, usually leukocytes, are collected by standard medically approved techniques and screened. Detection systems include ELISA procedures, binding of radiolabeled antibody to immobilized activated cells, flow cytometry, or other methods known to those skilled in the art. Inhibition of binding in the presence and absence of the lectin domain peptides can be used to detect defects or alterations in selectin binding. For selectins, such disorders would most likely be seen in patients with increased susceptibility to infections in which leukocytes would have defective binding to platelets and endothelium because of deficient leukocyte ligands for P-s lectin.

The peptide is labeled radioactively, with a fluorescent tag, enzymatically, or with electron dense material such as gold for electron microscopy. The cells to be examined, usually leukocytes, are incubated with the labeled peptides and binding assessed by methods described above with antibodies to P-selectin, or by other methods known to those skilled in the art. If ligands for P-selectin are also found in the plasma, they can also be measured with standard ELISA or radioimmunoassay procedures, using labeled P-selectin-derived peptide instead of antibody as the detecting reagent.

The peptides can also be useful in in vivo imaging of concentrations of cells bearing selectin ligands. Cells expressing selectin ligands whose abnormally high local concentrations or presence within the body, such as cancer cells, is indicative of a disorder, can be imaged using labeled peptides. These labels may be either intrinsic or extrinsic to the structure of the selectin-specific peptide and include, but are not restricted to, high energy emitters such as ^{111}In or non-radioactive dense atoms to enhance x-ray contrast.

The following examples are presented to illustrate, not limit, the invention. In the examples and throughout the specification, parts are by weight unless otherwise indicated.

EXAMPLE I: Glycyl-isoleucyl-tryptophyl-threonyl-tryptophyl-valine (SEQ ID. NO: 1)

The peptide was prepared by manual solid-phase synthesis using Boc chemistry using 1.6 mmol of TFA*Ile-Trp-Thr-Trp-Val-Resin (SEQ ID. NO: 8). 6 mmoles of Boc-Gly was activated by dicyclohexylcarbodiimide and hydroxybenzotriazole (6 mmoles of each) and coupled to the resin. Approximately 0.3 mmole of the TFA*Gly-Ile-Trp-Thr-Trp-Val-R sin (SEQ ID. NO: 9) was removed and dried in vacuo to yield 685 mg of peptide-resin.

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655 mg of the peptide-r sin was treated with 1 mL of anisole and 10 mL of HF for one hour at 0° to 4° C. The HF was removed by nitrogen stream and the resultant solids were triturated with diethyl ether (30 mL). The solids were
5 collected by filtration, washed with diethyl ether (3 x 10 mL) and then extracted with 50% TFA/methylene chloride (3 x 10 mL). The extracts were concentrated by use of a nitrogen stream in a fume hood. To the residue was added diethyl ether (~75 mL) to precipitate the crude peptide. The peptide
10 was collected via filtration, washed with ether (3 x 10 mL) and dried in vacuo over sodium hydroxide to yield 246 mg of crude peptide. 100 mg of the crude peptide had the Trp residues deformedylated by dissolution in 1% piperidine in 50% dimethylformamide/water (10 mL) and stirring for 2 hours at
15 room temperature. The deformylation solution was treated dropwise with acetic acid to adjust the pH to ~4.5. This solution was injected onto a Vydac 22 x 250 mm C18 10 µm particle sized 300 Angstrom pore packed column. Elution with a gradient of 20% to 60% B over 60 minutes at a flow rate of
20 10 mL/min was carried out (Solvent A = 0.1% TFA; Solvent B = 0.1% TFA in 80% Ethanol/Water). Fractions were collected and the appropriate ones pooled to give 53 mg of semi-pure peptide. Further purification was carried out by dissolution of the peptide in water (~20 mL) and addition of 30% ammonium
25 hydroxide dropwise. Four prep HPLC runs were performed using the above column and elution with isocratic 40% B buffer at 10 mL/min. Fractions were collected and the appropriate ones pooled to give 120 mg of white solid. Amino Acid Analysis: Gly 1.01 (1.00), Ile 0.97 (1.00), Thr 0.94 (1.00), Val 1.01
30 (1.00), Trp 1.84 (2.00), 10% peptide. FAB/MS: MH+ = 761.3 (MW = 760)

EXAMPLE II: Glycyl-glycyl-D-isoleucyl-D-tryptophyl-D-threonyl-D-tryptophyl-D-valine (SEQ ID. NO: 3)

The peptide was prepared by manual solid-phase
35 synthesis using Boc chemistry using 1.4 mmol of TFA*Gly-D-Ile-D-Trp-D-Thr-D-Trp-D-Val-PAM resin (SEQ ID. NO: 10). 5.2

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mm les of Boc-Gly was activated by dicyclohexylcarbodiimide and hydroxybenzotriazole (5.2 mmoles of each) and coupled to the resin. Approximately 0.3 mmol of the TFA-Gly-Gly-D-Ile-D-Trp-D-Thr-D-Trp-D-Val-PAM (SEQ ID. NO: 11) resin was removed and dried in vacuo to yield 881 mg of peptide-resin.

<u>WASH</u>	<u>REPETITIONS</u>	<u>TIME (min)</u>
30% TFA/CH ₂ Cl ₂	1	3
50% TFA/CH ₂ Cl ₂	1	16
CH ₂ Cl ₂	5	1
10 5% Diisopropylethylamine/ CH ₂ Cl ₂	1	4
CH ₂ Cl ₂	6	1

Coupling step (monitored by ninhydrin testing of a resin sample).

15 781 mg of the peptide-resin was treated with 1 mL of anisole and 10 mL of HF for one hour at 0° to 4° C. The HF was removed by nitrogen stream and the resultant solids were triturated with diethyl ether (30 mL). The solids were collected by filtration and washed with diethyl ether (3 x 10 mL) and then extracted with 50% TFA/methylene chloride (3 x 10 mL). The extracts were concentrated by use of a nitrogen stream in a fume hood. The residue was treated with diethyl ether (~75 mL) to precipitate the crude peptide. The peptide was collected via filtration, washed with ether (3 x 10 mL) and dried in vacuo over sodium hydroxide to yield 223 mg of crude peptide. 100 mg of the crude peptide had the Trp residues deacylated by dissolution in 1% piperidine in 50% dimethylformamide/water (20 mL) and stirring for 2 hours at room temperature. 4 x 5 mL injections were made onto a Vydac 20 22 x 250 mm C18 10 µm particle sized 300 Angstrom pore packed column. Elution with a gradient of 30% to 45% B over 30 minutes at a flow rate of 10 mL/min was carried out (Solvent A = 0.1% TFA; Solvent B = 0.1% TFA in 80% Ethanol/Water). Fractions were collected and the appropriate ones pooled to 35 give 38 mg of white solid. Amino Acid Analysis: Gly 1.99

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(2.00), Ile 0.84 (1.00), Thr 0.83 (1.00), Val 1.00 (1.00),
Trp 1.76 (2.00), 74% peptide. FAB/MS MH+ = 818 (MW = 817)
EXAMPLE III: Glycyl-glycyl-isoleucyl-tryptophyl-threonyl-tryptophyl-valine (SEQ ID. NO: 2)

5 The peptide was prepared by manual solid-phase
synthesis using Boc chemistry using 1.3 mmol of TFA-Gly-Ile-
Trp-Thr-Trp-Val-Resin (SEQ ID. NO: 9). 5 mmoles of Boc-Gly
was activated by dicyclohexylcarbodiimide and
hydroxybenzotriazole (5 mmoles of each) and coupled to the
10 resin. Approximately 0.3 mmole of the TFA-Gly-Gly-Ile-Trp-
Thr-Trp-Val-Resin (SEQ ID. NO: 12) was removed and dried in
vacuo to yield 685 mg of peptide-resin.

655 mg of the peptide-resin was treated with 1 mL
of anisole and 10 mL of HF for one hour at 0° to 4° C. The HF
15 was removed by nitrogen stream and the resultant solids were
trituated with diethyl ether (30 mL). The solids were
collected by filtration, washed with diethyl ether (3 x 10
mL) and then extracted with 70% acetic acid (3 x 10 mL). The
extracts were combined and lyophilized to yield 128 mg of
20 crude peptide. 84 mg of the crude peptide had the Trp
residues deprotected by dissolution in 2% piperidine (10 mL)
and stirring for 2 hours at room temperature. This solution
was injected over three runs onto a Vydac 22 x 250 mm C18 10
µm particle sized 300 Angstrom pore packed column. Elution
25 with a gradient of 30% to 50% B over 30 minutes at a flow
rate of 10 mL/min was carried out (Solvent A = 0.1% TFA;
Solvent B = 0.1% TFA in 80% Ethanol/Water). Fractions were
collected and the appropriate ones pooled to give 53 mg of
white solid.

30 Amino Acid Analysis: Gly 2.00 (2.00), Ile 0.92 (1.00), Thr
0.90 (1.00), Val 1.00 (1.00), Trp 1.79 (2.00), 61% peptide
FAB/MS: MH+ = 818 (MW = 817)

EXAMPLE IV: Glycyl-D-isoleucyl-D-tryptophyl-D-threonyl-D-tryptophyl-D-valine (SEQ ID. NO: 4)

35 The peptide was prepared by manual solid-phase
synthesis using Boc chemistry using 1.7 mmol of TFA-D-Ile-D-

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Trp-D-Thr-D-Trp-D-Val-PAM resin (SEQ ID. NO: 13). 6.8 mmoles of Boc-Gly was activated by dicyclohexylcarb diimide and hydroxybenzotriazol (6.8 mmoles of each) and coupled to the resin. Approximately 0.3 mmole of the TFA*Gly-D-Ile-D-Trp-D-
 5 Thr-D-Trp-D-Val-PAM resin (SEQ ID. NO: 10) was removed and dried in vacuo to yield 843 mg of peptide-resin. The wash sequence used was as follows:

	<u>WASH</u>	<u>REPETITIONS</u>	<u>TIME (mins)</u>
	30% TFA/CH ₂ Cl ₂	1	3
10	50% TFA/CH ₂ Cl ₂	1	16
	CH ₂ Cl ₂	5	1
	5% Diisopropylethylamine/ CH ₂ Cl ₂	1	4
	CH ₂ Cl ₂	6	1

15 Coupling step (monitored by ninhydrin testing of a resin sample).

781 mg of the peptide-resin was treated with 1 mL of anisole and 10 mL of HF for one hour at 0° to 4° C. The HF was removed by nitrogen stream and the resultant solids were
 20 triturated with diethyl ether (30 mL). The solids were collected by filtration and washed with diethyl ether (3 x 10 mL) and then extracted with 70% acetic acid (3 x 10 mL). The extracts were combined and lyophilized to yield 171 mg of crude peptide. 100 mg of the crude peptide had the Trp
 25 residues deformylated by dissolution in 1% piperidine in 50% dimethylformamide/water (20 mL) and stirring for 2 hours at room temperature. The deformylation solution was injected onto a Vydac 22 x 250 mm C18 10 µm particle sized 300
 Angstrom pore packed column over four runs. Elution with a
 30 gradient of 30% to 50% B over 40 minutes at a flow rate of 10 mL/min was carried out (Solvent A = 0.1% TFA; Solvent B = 0.1% TFA in 80% Ethanol/Water). Fractions were collected and the appropriate ones pooled to give 114 mg of white solid.
 Amino Acid Analysis: Gly 1.04 (1.00), Ile 0.93 (1.00), Thr
 35 0.91 (1.00), Val 1.03 (1.00), Trp 1.89 (2.00), 12% peptide.
 FAB/MS: MH⁺ = 761.1 (MW = 759)

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EXAMPLE V: Lysyl-thre nyl-tryptophyl-thr nyl-tryptophyl-valin -amid (SEQ ID. NO: 6)

The peptide was prepar d on an ABI Model 431A Peptide Synthesizer using Version 1.12 of the standard BOC software. 4-methyl benzhydrylamine resin (625 mg, 0.5 mmol) was used in the synthesis. The final weight of the resin was 1.12 g.

The peptide was cleaved from the resin (1.12 g) using 11 mL of HF and 1.1 mL of anisole for 60 min at 0°C. The resin was washed with ether and the peptide extracted with 25% acetic acid to give 324 mg of crude peptide.

The crude peptide (324 mg) was purified on a Vydac C-18 column (15 μ , 5 x 25 cm) eluting with a 25-50% gradient of 80% acetonitrile in 0.1% TFA over 120 min at a flow rate of 15 mL per min. Fractions were collected, analyzed by HPLC and pure fractions pooled and lyophilized to give 128 mg of pure peptide. Amino acid analysis: Lys 1.02 (1), Thr 1.82 (2), Trp 1.83 (2), Val 0.98 (1). FAB/MS: MH⁺ 819.7

EXAMPLE VI: Threonyl-tryptophyl-threonyl-tryptophyl-valine- amide (SEQ ID. NO: 7)

The peptide was prepared on an ABI Model 431A Peptide Synthesizer using Version 1.12 of the standard BOC software. 4-methyl benzhydrylamine resin (625 mg, 0.5 mmol) was used in the synthesis. The final weight of the resin was 1.0 g.

The peptide was cleaved from the resin (1.0 g) using 10 mL of HF and 1 mL of anisole for 60 min at 0°C. The resin was washed with ether and the peptide extracted with 35% acetic acid to give 287 mg of crude peptide.

The crude peptide (287 mg) was purified on a Vydac C-18 column (15 μ , 5 x 25 cm) eluting with a 25-45% gradient of 80% acetonitrile in 0.1% TFA over 120 min at a flow rate of 15 mL per min. Fractions were collected, analyzed by HPLC and pure fractions pooled and lyophilized to give 126 mg of pure peptide. Amino acid analysis: Thr 1.82 (2), Trp 1.85 (2), Val 1.00 (1). FAB/MS: MH⁺ 691.5

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EXAMPLE VII: Asparaginyl-lysyl-threonyl-tryptophyl-thr onyl-trypt phyl-valin -amid (SEQ ID. NO: 5)

The p ptid was prepared on an ABI Model 431 peptid synthesizer using V rsion 1.12 of the standard BOC software. 4-methylbenzhydrylamine resin (0.588 mg, 0.5 mmol) was used in the synthesis.

The peptide was cleaved from the resin using 20 mL of HF and 2 mLs of anisole for 60 min at 0°C. The resin was washed with ether and the peptide extracted with a 50% solution of aqueous acetic acid.

The crude peptide was purified on a Vydac C-18 column (15 μ , 5 x 25 cm) eluting with a 25-45% gradient of 80% acetonitrile and 0.1% TFA over 120 min at a flow rate of 15 mL per min. Fractions were collected, analyzed by HPLC and pure fractions pooled and lyophilized to give 92 mg. Amin acid analysis: Asx 1.00 (1), Lys 1.00 (1), Thr 1.66 (2), Trp 0.71 (2), Val 1.00 (1). FAB/MS: MH⁺ 933.3 (933.09).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Heavner, George A.
Riexinger, Douglas
Mervic, Miljenko
- (ii) TITLE OF INVENTION: Peptide Inhibitors of Selectin Binding
- (iii) NUMBER OF SEQUENCES: 13
- 10 (iv) CORRESPONDENCE ADDRESS:
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15 (D) STATE: Pennsylvania
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(F) ZIP: 19103
- (v) COMPUTER READABLE FORM:
20 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
25 (A) APPLICATION NUMBER: US 891,986
(B) FILING DATE: 05-MAY-1992
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
30 (A) NAME: Elderkin, Dianne B.
(B) REGISTRATION NUMBER: 28,598
(C) REFERENCE/DOCKET NUMBER: CCOR-0024
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(C) TELEX: 710-670-1334

35 (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- 40 (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO

- 32 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Ile Trp Thr Trp Val
1 5

(2) INFORMATION FOR SEQ ID NO:2:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

10 (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Gly Ile Trp Thr Trp Val
1 5

(2) INFORMATION FOR SEQ ID NO:3:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20 (iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /label= D-Ile
/note= "The third residue is D-Ile"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 4
- (D) OTHER INFORMATION: /label= D-Trp
/note= "The fourth residue is D-Trp"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /label= D-Thr
/note= "The fifth residue is D-Thr"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site

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(B) LOCATION: 6
(D) OTHER INFORMATION: /label= D-Trp
/note= "The sixth residue is D-Trp"

(ix) FEATURE:

5 (A) NAME/KEY: Modified-site
(B) LOCATION: 7
(D) OTHER INFORMATION: /label= D-Val
/note= "The seventh residue is D-Val"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

10 Gly Gly Xaa Xaa Xaa Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:4:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

20 (A) NAME/KEY: Modified-site
(B) LOCATION: 2
(D) OTHER INFORMATION: /label= D-Ile
/note= "The second residue is D-Ile"

(ix) FEATURE:

25 (A) NAME/KEY: Modified-site
(B) LOCATION: 3
(D) OTHER INFORMATION: /label= D-Trp
/note= "The third residue is D-Trp"

(ix) FEATURE:

30 (A) NAME/KEY: Modified-site
(B) LOCATION: 4
(D) OTHER INFORMATION: /label= D-Thr
/note= "The fourth residue is D-Thr"

(ix) FEATURE:

35 (A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /label= D-Trp
/note= "The fifth residue is D-Trp"

(ix) FEATURE:

40 (A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /label= D-Val

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/note= "The sixth residue is D-Val"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Xaa Xaa Xaa Xaa Xaa
1 5

5 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 7

15 (D) OTHER INFORMATION: /label= Val-NH2

/note= "The carboxy terminal amino acid,
Val, is amidated"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn Lys Thr Trp Thr Trp Xaa
20 1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

25 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: Modified-site

30 (B) LOCATION: 6

(D) OTHER INFORMATION: /label= Val-NH2

/note= "The carboxy terminal amino acid,
Val, is amidated"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

35 Lys Thr Trp Thr Trp Xaa

- 35 -

1

5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- 10 (A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /label= Val-NH2
/note= "The carboxy terminal residue, Val,
is amidated"

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Thr Trp Thr Trp Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

25 (ix) FEATURE:

- (A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /label= Val-resin
/note= "The carboxy terminal amino acid,
30 Val, is attached to a resin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ile Trp Thr Trp Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:9:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids

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(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

5 (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 6

(D) OTHER INFORMATION: /label= Val-resin

10 Val, is attached to a resin"
/note= "The carboxy terminal amino acid,

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Ile Trp Thr Trp Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:10:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20 (iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 2

25 (D) OTHER INFORMATION: /label= D-Ile
/note= "The second residue is D-Ile"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 3

30 (D) OTHER INFORMATION: /label= D-Trp
/note= "The third residue is D-Trp"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 4

35 (D) OTHER INFORMATION: /label= D-Thr
/note= "The fourth residue is D-Thr"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 5

40 (D) OTHER INFORMATION: /label= D-Trp
/note= "The fifth residue is D-Trp"

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- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 6
 (D) OTHER INFORMATION: /label= D-Val-resin
5 /note= "The carboxy terminal residue, D-Val,
is attached to a resin"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

 Gly Xaa Xaa Xaa Xaa Xaa
 1 5
- 10 (2) INFORMATION FOR SEQ ID NO:11:

 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
- 15 (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 3
20 (D) OTHER INFORMATION: /label= D-Ile
 /note= "The third residue is D-Ile"
- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 4
25 (D) OTHER INFORMATION: /label= D-Trp
 /note= "The fourth residue is D-trp"
- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 5
30 (D) OTHER INFORMATION: /label= D-Thr
 /note= "The fifth residue is D-Thr"
- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 6
35 (D) OTHER INFORMATION: /label= D-Trp
 /note= "The sixth residue is D-Trp"
- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 7
40 (D) OTHER INFORMATION: /label= D-Val-resin
 /note= "The carboxy terminal residue, D-Val,
is attached to a resin"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Gly Xaa Xaa Xaa Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:12:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

10 (iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 7
(D) OTHER INFORMATION: /label= Val-resin
15 /note= "The carboxy terminal residue, Val,
is attached to a resin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Gly Ile Trp Thr Trp Xaa
1 5

20 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 1
30 (D) OTHER INFORMATION: /label= D-Ile
/note= "The first residue is D-Ile"

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 2
35 (D) OTHER INFORMATION: /label= D-Trp
/note= "The second residue is D-trp"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

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(B) LOCATION: 3
(D) OTHER INFORMATION: /label= D-Thr
/note= "The third residue is D-Thr"

(ix) FEATURE:

5 (A) NAME/KEY: Modified-site
(B) LOCATION: 4
(D) OTHER INFORMATION: /label= D-Trp
/note= "The fourth residue is D-Trp"

(ix) FEATURE:

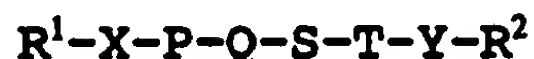
10 (A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /label= D-Val-resin
/note= "The carboxy terminal residue, D-Val,
is attached to a resin"

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Xaa Xaa Xaa Xaa Xaa
1 5

WHAT IS CLAIMED IS:

1. Peptides of the formula:



(I)

5 or pharmaceutically acceptable salts thereof, where:

X is an N-terminus amino acid linear chain of from zero to 10 amino acids, and R^1 is a moiety attached to the terminal α -amino group of X or the α -amino group of P if X is zero, or the α -amino group of Q if X is zero and P is

10 desamino acid;

Y is a C-terminus amino acid linear chain of from zero to 10 amino acids, and R^2 is a moiety attached to the carbon of the carboxyl function of Y ($C(O)R^2$) or the carbon of the carboxyl function of T if Y is zero;

15 P is D- or L-lysine, D- or L- ϵ -acetyl-lysine, D- or L- asparagine, glycine, D- or L-valine, D- or L-glutamine, D- or L-glutamic acid, D- or L-alanine, or a desamino acid; Q is an amino acid selected from the group consisting of D- or L-threonine, D- or L-isoleucine, D- or L-valine, D- or L-
20 alanine, D- or L-glutamine;

S is an amino acid selected from the group consisting of consisting of D- or L-tryptophan, D- or L-glutamine, D- or L-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, D- or L- ϵ -acetyl-lysine;

25 T is an amino acid selected from the group consisting of D- or L-threonine, D- or L-valine, D- or L-alanine, D- or L-glutamine, D- or L- ϵ -acetyl-lysine;

R^1 is hydrogen, lower alkyl, aryl, formyl, alkanoyl, aroyl, alkyloxycarbonyl or arroyloxycarbonyl;

30 R^2 is OH, OR^3 , where R^3 is lower alkyl or aryl, or R^2 is NR^4R^5 , where R^4 and R^5 are each selected independently from hydrogen, lower alkyl, aryl or cyclic alkyl;

provided that X is not equal to Z-A-B-, where Z is a linear sequence of 0-8 amino acids; A is an amino acid

35 selected from the group consisting of D- or L-asparagine, D- or L-isoleucine, and D- or L-valine; and B is an amino acid

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selected from the group consisting of D- or L-asparagine and glycine.

2. The peptide of Claim 1 wherein X is selected from the group consisting of Asn, D-Asn, Gly-Gln, D-Gln, and ϵ -acetyl-lysine.

3. The peptide of Claim 1 wherein Y is selected from the group consisting of Gln-Val, Gln-D-Val, D-Gln-Val, D-Gln-D-Val, Trp-Gln, D-Trp-Gln, Trp-D-Gln, D-Trp-D-Gln, 7-hydroxy-1,2,3,4-tetrahydroisoquiniline-3-carboxylic acid-Gln, D-7-hydroxy-1,2,3,4-tetrahydroisoquiniline-3-carboxylic acid-Gln, D-7-hydroxy-1,2,3,4-tetrahydroisoquiniline-3-carboxylic acid-Gln, D-7-hydroxy-1,2,3,4-tetrahydroisoquiniline-3-carboxylic acid-D-Gln, ϵ -acetyl-lysine-Gln, D- ϵ -acetyl-lysine-Gln, ϵ -acetyl-lysine-D-Gln, D- ϵ -acetyl-lysine-D-Gln.

4. A peptide of Claim 1 selected from the group consisting of peptides having the formula:

Gly-Gly-D-Ile-D-Trp-D-Thr-D-Trp-D-Val (SEQ ID. NO: 3)

Gly-D-Ile-D-Trp-D-Thr-D-Trp-D-Val (SEQ ID. NO: 4)

Gly-Ile-Trp-Thr-Trp-Val (SEQ ID. NO: 1)

Gly-Gly-Ile-Trp-Thr-Trp-Val (SEQ ID. NO: 2)

Asn-Lys-Thr-Trp-Thr-Trp-Val-NH₂ (SEQ ID. NO: 9)

Lys-Thr-Trp-Thr-Trp-Val-NH₂ (SEQ ID. NO: 6)

Thr-Trp-Thr-Trp-Val-NH₂ (SEQ ID. NO: 7)

5. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and an effective amount of one or more peptides of Claim 1.

6. A method for inhibiting leukocyte adherence in a human patient comprising administering to said patient an effective quantity of a peptide of Claim 1.

7. A method for modifying binding of a selectin in a human patient comprising administering to said patient an effective quantity of a peptide of Claim 1.

8. A method of Claim 7 wherein said selectin is selected from the group consisting of P-selectin, E-selectin and L-selectin.

9. A method of treating a human patient in need of treatment for inflammation comprising administering to said patient an effective quantity of a peptide of Claim 1.

10. A method of treating a human patient in need of treatment for coagulation comprising administering to said patient an effective quantity of a peptide of Claim 1.

11. A method of treating a human patient for a condition selected from the group consisting of ischemia and reperfusion, bacterial sepsis and disseminated intravascular coagulation, adult respiratory distress syndrome, tumor metastasis, rheumatoid arthritis and atherosclerosis, comprising administering to said patient an effective quantity of a peptide of Claim 1.

12. A method of detecting defective selectin-binding ligands in a human patient comprising contacting cells from said patient with a labeled peptide of Claim 1, and assessing the binding of said labeled peptide to said cells.

13. A method of Claim 12 wherein said cells are leukocytes.

14. A method of Claim 12 wherein said peptide is labeled with a moiety selected from the group consisting of radioactive tracers, fluorescent tags, enzymes, and electron-dense materials.

15. A method of detecting defective selectin-binding ligands in a human patient comprising contacting cells from said patient with a labeled peptide of Claim 1,

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and assessing the binding of said labeled peptide to said cells.

16. A method of Claim 15 wherein said cells are leukocyte.

5 17. A method of Claim 15 wherein said cells are tumor cells.

18. A method of Claim 15 wherein said peptide is labeled with a moiety selected from the group consisting of
10 radioactive tracers, fluorescent tags, enzymes, and electron-dense materials.

19. A method of preparing a peptide of Claim 1 comprising adding amino acids either singly or in pre-formed blocks of amino acids to an appropriately functionalized
15 solid support.

20. The method of Claim 1 wherein said amino acids are assembled either singly or in preformed blocks in solution or suspension by chemical ligation techniques.

21. The method of preparing a peptide of Claim 1
20 comprising amino acids either singly or in pre-formed blocks in solution or suspension by enzymatic ligation techniques.

22. The method of preparing a peptide of Claim 1 wherein said peptide is produced enzymatically by inserting nucleic acid encoding the peptide into an expression vector,
25 expressing the DNA, and translating the DNA into the peptide.

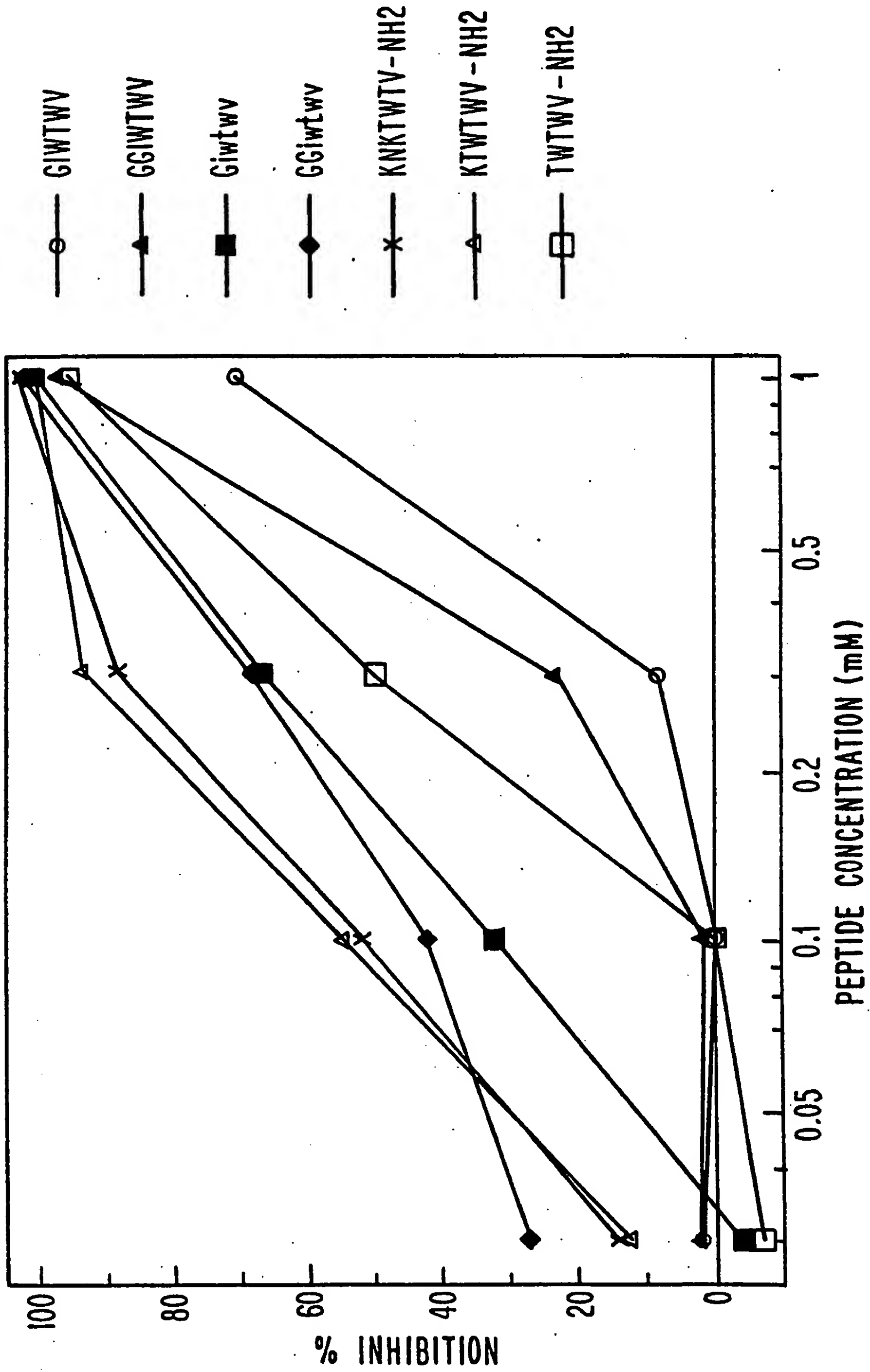


Fig. 1

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : CO7K 07/06

US CL : 530/329, 330; 514/016, 017

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/329, 330; 514/016, 017

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS online, medline, aps

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
X,P	US, A, 5,198,424 (McEver) 30 March 1993, see entire document	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

- | | | | |
|-----|---|-----|--|
| *A* | document defining the general state of the art which is not considered to be part of particular relevance | *T* | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *E* | earlier document published on or after the international filing date | *X* | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *L* | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *Y* | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *O* | document referring to an oral disclosure, use, exhibition or other means | *Z* | document member of the same patent family |
| *P* | document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search
30 July 1993Date of mailing of the international search report
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